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The Exonuclease Domain of Lassa Virus Nucleoprotein Is Involved in Antigen-Presenting-Cell-Mediated NK Cell Responses

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ABSTRACT

Lassa virus is an Old World *Arenavirus* which causes Lassa hemorrhagic fever in humans, mostly in West Africa. Lassa fever is an important public health problem, and a safe and effective vaccine is urgently needed. The infection causes immunosuppression, probably due to the absence of activation of antigen-presenting cells (dendritic cells and macrophages), low type I interferon (IFN) production, and deficient NK cell function. However, a recombinant Lassa virus carrying D389A and G392A substitutions in the nucleoprotein that abolish the exonuclease activity and IFN activation loses its inhibitory activity and induces strong type I IFN production by dendritic cells and macrophages. We show here that during infection by this mutant Lassa virus, antigen-presenting cells trigger efficient human NK cell responses *in vitro*, including production of IFN-γ and cytotoxicity. NK cell activation involves close contact with both antigen-presenting cells and soluble factors. We report that infected dendritic cells and macrophages express the NKG2D ligands major histocompatibility complex (MHC) class I-related chains A and B and that they may produce interleukin-12 (IL-12), IL-15, and IL-18, all involved in NK cell functions. NK cell degranulation is significantly increased in cocultures, suggesting that NK cells seem to kill infected dendritic cells and macrophages. This work confirms the inhibitory function of Lassa virus nucleoprotein. Importantly, we demonstrate for the first time that Lassa virus nucleoprotein is involved in the inhibition of antigen-presenting cell-mediated NK cell responses.

IMPORTANCE

The pathogenesis and immune responses induced by Lassa virus are poorly known. Recently, an exonuclease domain contained in the viral nucleoprotein has been shown to be able to inhibit the type I IFN response by avoiding the recognition of viral RNA by cell sensors. Here, we studied the responses of NK cells to dendritic cells and macrophages infected with a recombinant Lassa virus in which the exonuclease functions have been abolished and demonstrated that NK cells are strongly activated and presented effective functions. These results show that the strategy developed by Lassa virus to evade innate immunity is also effective on NK cells, explaining the weak NK cell activation observed with the wild-type virus. By providing a better understanding of the interactions between Lassa virus and the host immune system, these results are important for the field of arenavirus biology and may be useful for a vaccine approach against Lassa fever.

Lassa fever is a public health threat in West Africa. There are 300,000 to 500,000 cases annually and 5,000 to 6,000 deaths (1). The transmission of Lassa virus (LASV), the etiologic agent, to humans can cause a wide variety of symptoms ranging from an asymptomatic infection to a fatal severe hemorrhagic fever in up to 15% of cases. The antiviral drug ribavirin is effective, but there are issues associated with its use, particularly, cost, the need for early administration, and the deleterious effects observed during pregnancy. A safe and effective vaccine is urgently needed, and several vaccine candidates have been studied (2). However, no useful vaccine is commercially available and approved.

Lassa virus is a bisegmented, negative, single-stranded RNA (ssRNA) virus belonging to the Old World *Arenavirus* family (3). The viral genome encodes four genes in an ambisense manner. The small (S) segment encodes the precursor of the glycoprotein (GPC, cleaved to give GP1 and GP2) and the nucleoprotein (NP). The RNA-dependent RNA polymerase L and the small zinc finger matrix Z protein are encoded by the large (L) segment. NP is a multifunctional protein involved in viral genomic RNA encapsidation, viral RNA synthesis, and, by inhibiting the type I interferon (IFN) pathway, immune evasion (4–6). NP has a 3′-5′ exonuclease activity comparable to the DEDDh enzymes so that it can process double-stranded RNA (dsRNA) substrates (7–9). The degradation of immunostimulatory dsRNA molecules prevents RIG-I (retinoic acid-inducible gene I) recognition and downstream initiation of type I IFN production (7, 10).

LASV replicates in antigen-presenting cells (APC), including dendritic cells (DC) and macrophages (Mϕ), without causing cytopathic effects (11, 12). Upon infection, DC remain unactivated, and Mϕ produce only very small amounts of type I IFN (13). Low and late T cell responses without cytotoxicity or memory occur during LASV infection of DC in an *in vitro* model (14). Similarly, we have shown that LASV-infected DC do not induce NK cell activation *in vitro* (15). LASV infection of Mϕ leads to the activation of NK cells, the downregulation of the chemokine receptor

Received 2 July 2014 **Accepted** 16 September 2014

Published ahead of print 24 September 2014

Editor: S. Perlman

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doi:10.1128/JVI.01908-14

CXCR3, the upregulation of the cytotoxicity receptor NKp30, and an increased ability to kill sensitive K562 targets. The activation mediated by LASV-infected Mϕ, however, is not sufficient to enable the killing of infected cells or the production of IFN-γ. We also found that NK cell activation requires type I IFN although only small amounts are produced.

NK cell functions during viral infections have been extensively studied (16). NK cells are involved in viral clearance by killing infected cells and in the initiation of T cell responses promoted by IFN-γ production (17). The cross talk with APC potentiates NK cell functions: receptor/ligand signaling during contacts between cells and with soluble mediators such as type I IFN are essential for the activation of NK cell cytotoxicity and trigger NK cell-mediated production of IFN-γ (18).

We have generated a recombinant LASV containing D389A and G392A substitutions in the C-terminal domain of NP (rNP-LASV). D389 was previously shown to be involved in the exonuclease activity of NP as it is within the active site, and G392 was found to be crucial for IFN suppression (4, 7, 8). rNP-LASV, but not the recombinant wild-type virus (rWT-LASV), induces substantial production of type I IFN by DC and Mϕ (19). We show here that DC and Mϕ infected by rNP-LASV induce strong NK cell activation leading to IFN-γ secretion. The stimulated NK cells trigger cytotoxicity toward infected cells and activation of APC. This work shows for the first time that the exonuclease function of LASV NP is involved in the inhibition of APC functions, including mediating NK cell activation. NK cells are central to the initiation of T cell responses, so these findings contribute insights that will help in the design of vaccines that elicit long-lasting T cell immunity.

MATERIALS AND METHODS

Cells and virus strains. Vero E6 and K562 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin-streptomycin and with 5% and 10% fetal calf serum (FCS), respectively (all from Life Technologies, Saint Aubin, France), at 37°C with 5% CO_2.

Recombinant wild-type LASV (rWT-LASV) and NP-D389A/G392A (rNP-LASV) were generated by reverse genetics as previously described (19) and passaged twice in Vero E6 cells. Viral supernatants were harvested, titrated, and used as the infectious virus stock. Virus-free supernatants of Vero E6 cell cultures were used for mock experiments.

Cell lines and virus stocks were not contaminated by mycoplasma.

All experiments were carried out in biosafety level 4 (BSL4) facilities (Laboratoire P4 Jean Mérieux-Inserm, Lyon, France).

Preparation of DC, Mϕ, and NK cells. Monocytes and NK cells were isolated from the blood of consenting healthy donors provided by the Etablissement Français du Sang (Lyon, France) as previously described (11, 15). In particular, monocytes and NK cells were purified by immunomagnetic depletion and negative selection with a monocyte isolation kit and an NK cell isolation kit, respectively (both from Miltenyi Biotech, Auburn, CA, USA).

Monocytes were then induced to differentiate into Mϕ and DC by culture in RPMI 1640 medium with GlutaMAX I, 1% penicillin-streptomycin, 10 mM HEPES, 1% nonessential amino acids, and 10% FCS (all from Life Technologies) (C-RPMI), supplemented with 50 ng/ml macrophage colony-stimulating factor (M-CSF; Miltenyi Biotech) and 10% autologous decomplemented plasma for Mϕ or with 1,000 IU/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 500 IU/ml interleukin-4 (IL-4) (all from PeproTech, London, United Kingdom) for DC; 40% of the medium and cytokines was replaced every 48 h, and DC and Mϕ were harvested after 6 days.

NK cells were frozen in 90% FCS–10% dimethyl sulfoxide (DMSO) (Sigma, Saint-Quentin Fallavier, France) and stored in liquid nitrogen until culture with DC or Mϕ.

Infection of DC and Mϕ and culture with NK cells. DC and Mϕ were incubated for 1 h at 37°C with virus-free Vero E6 cell supernatant (mock) or recombinant LASV at a multiplicity of infection (MOI) of 2, washed, and resuspended at 10^6 cells/ml in C-RPMI medium.

In coculture experiments, NK cells were thawed and added to APC cultures at an NK cell/APC ratio of 1:5. In some experiments, contact between NK cells and APC was prevented by a polycarbonate membrane with 0.4-μm pores (Corning Life Sciences, Schiphol-Rijk, The Netherlands).

Phenotypic analysis by flow cytometry. Cells were harvested and incubated with specific monoclonal antibody (MAb) in phosphate-buffered saline (PBS)–5% human serum for 30 min at 4°C. NK cell surface molecules were stained with the following antibodies (Abs): anti-CD25-fluorescein isothiocyanate (FITC) or allophycocyanin (APC)-Cy7 (M-A251), anti-CD56-Alexa Fluor 488 or phycoerythrin (PE)-Cy5 (B159), anti-CD69-PE-Cy5 (FN50), and anti-CXCR3-PE-Cy5 (1C6/CXR3) from BD Pharmingen (San Diego, CA, USA); anti-CD3-PE-Cy7 (SK7) or Krome Orange (UCHT1), anti-CD25-FITC (B1.49.9), anti-NKp30-PE (Z25), and anti-NKG2D-PE (ON72) from Beckman Coulter (Marseille, France). For analysis of intracellular granzyme B (GrzB), NK cells were permeabilized with a Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer's instructions and stained with anti-GrzB-FITC (GB11; BD Pharmingen). Similarly, DC and Mϕ were stained with anti-major histocompatibility complex (MHC) class I-related chains A and B (MICA/B)-PE (6D4; BD Pharmingen) and anti-Fas-PE-Cy5 (DX2; eBioscience, Paris, France).

Finally, cells were fixed with paraformaldehyde in PBS and analyzed by flow cytometry (Epics-XL and Gallios flow cytometers; Beckman Coulter). Data were computed with FlowJo software. NK cells were gated according to forward scatter and side scatter characteristics and to a $CD3^-$ $CD56^+$ phenotype.

CD107a assay. NK cell degranulation was assessed by CD107a labeling. Mouse anti-CD107a-FITC Ab (H4A3; BD Pharmingen) was added at 48 h postinfection, and cells were incubated for 4 h at 37°C. Monensin (Golgi-Stop; BD Pharmingen) was used for the last 3 h to prevent CD107a degradation. NK cells were then labeled using Ab specific for phenotypic surface molecules and analyzed by flow cytometry. NK cells were gated as $CD56^+$ $CD3^-$ $CD14^-$ cells. In some experiments, K562 target cells were added after 48 h to the NK/APC cultures at an effector/target (E/T) ratio of 10:1 to induce degranulation.

ELISA. Supernatants of cultures were collected at 24 and 72 h postinfection and stored at −80°C. Commercial enzyme-linked immunosorbent assay (ELISA) kits were used to assay IFN-γ (Bender MedSystems), soluble UL16-binding protein 1 (sULBP-1), and IL-18 (R&D Systems, Lille, France) according to the manufacturers' instructions.

Analysis of mRNA levels by quantitative reverse transcription-PCR (RT-PCR). Total RNA was obtained at 24 h after infection using RNeasy kits and DNA I digestion (Qiagen, Hilden, Germany). Reverse transcription was carried out using SuperScript III reverse transcriptase, RNaseOUT, first-strand buffer, dithiothreitol (DTT), oligo(dT), and deoxynucleoside triphosphate (dNTP) mix (all from Life Technologies). The resulting cDNA was analyzed by real-time PCR in an ABI Prism 7000 (Applied Biosystems, Foster City, CA, USA) or a LightCycler 480 (Roche Diagnostic, Meylan, France) thermocycler with TaqMan Universal master mix and commercial primers and probes for the sequences encoding IFN-γ, GrzB, perforin, FasL, TRAIL, MICA, MICB, ULBP-1, ULBP-2, B7H6, IL-12p35, IL-12p40, IL-15, and IL-18 (all from Applied Biosystems). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified with commercial primers and probes (Applied Biosystems) for normalization. Relative mRNA levels were then calculated as $2^{-\Delta CT}$, where C_T is cycle threshold and $\Delta C_T = C_T$ of the target gene $- C_T$ of GAPDH.

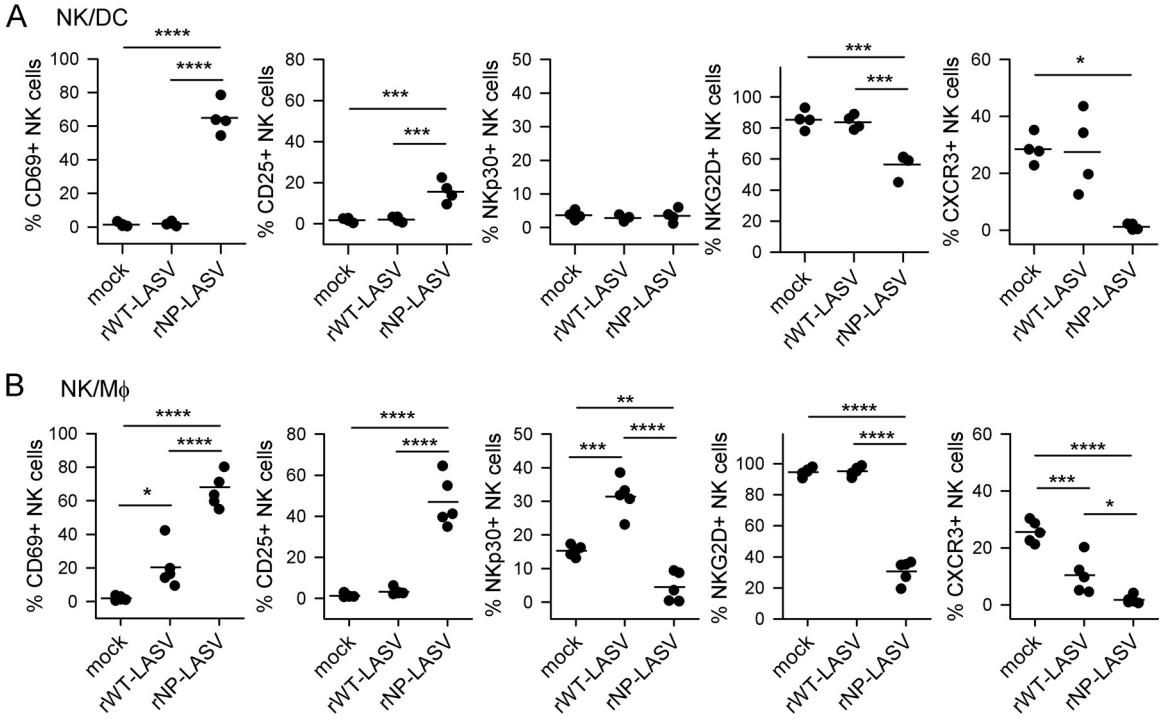

FIG 1 The infection of DC and Mϕ by rNP-LASV induces NK cell activation and modification of the receptor repertoire. Resting NK cells were cocultured with mock-, rWT-LASV-, or rNP-LASV-infected DC (A) or Mϕ (B), and the expression of CD69, CD25, NKp30, NKG2D, and CXCR3 was analyzed at 72 h postinfection by flow cytometry. NK cells were gated according to forward scatter/side scatter characteristics and the expression of CD56. The results are reported as percentages of positive cells from independent experiments ($n = 4$ and $n = 5$ for NK/DC and NK/Mϕ experiments, respectively), indicated by dots, and mean values are indicated by horizontal bars. Statistical significance of differences was determined using one-way ANOVA, and differences between conditions were assessed by a Bonferroni *post hoc* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

Statistical analysis. Statistical analyses were performed with GraphPad Prism software, version 5.04 (San Diego, CA, USA). One-way analysis of variance (ANOVA) with a Bonferroni *post hoc* test, Kruskal-Wallis with Dunn's test, and two-way ANOVA followed by a Bonferroni test were used as specified in the figure legends to determine the significance of differences ($P < 0.05$).

RESULTS

NK cells are activated by rNP-LASV-infected APC. As previously shown (19), we observed that in contrast to rWT-LASV, rNP-LASV was strongly attenuated in DC and Mϕ single cultures as well as NK/APC cocultures (data not shown). Infection of APC with rNP-LASV leads to the production of large amounts of type I IFN, whereas this is not the case following infection with rWT-LASV (19). Similarly, the levels of type I IFN produced in NK/APC cocultures were comparable to the level found in APC single cultures (data not shown). We infected DC and Mϕ with rWT-LASV, rNP-LASV, or a virus-free supernatant (mock), and NK cells were then added. The cells were incubated for 72 h, and the NK cells were stained for various markers of activation and analyzed by flow cytometry. During rWT-LASV infection, only Mϕ were able to induce CD69 expression on NK cells, and the level of CD25 expression remained unchanged (Fig. 1). CD69 and CD25 were significantly upregulated on NK cells after stimulation with rNP-LASV-infected DC and Mϕ (Fig. 1). The numbers of CD69-expressing NK cells were higher during rNP-LASV than rWT-LASV infection of Mϕ. Thus, the infection of DC and Mϕ by a recombinant LASV mutated within or nearby the exonuclease domain of NP leads to strong NK cell activation.

Activating and chemokine NK cell receptors are modulated by rNP-LASV-infected APC. NK cells were stimulated with rWT-LASV-infected, rNP-LASV-infected, or mock-infected DC or Mϕ for 72 h, and the expression of selected NK cell receptors was investigated by flow cytometry. We analyzed the expression of NKp30 and NKG2D, which are involved in NK cell-mediated cytotoxicity. NKp30 was significantly upregulated on NK cells cultured with rWT-LASV-infected Mϕ only, and the expression of NKG2D was unchanged upon culture with rWT-LASV-infected Mϕ (Fig. 1). NKp30 expression was not modified during rNP-LASV infection of DC, but it was significantly decreased after stimulation with infected Mϕ. rNP-LASV-infected DC and Mϕ induced NKG2D downregulation on NK cells. We also studied the expression of the chemokine receptor CXCR3. Upon rWT-LASV infection, CXCR3 was downregulated only after stimulation with infected Mϕ (Fig. 1). In contrast, rNP-LASV-infected DC and Mϕ each induced a significant and large decrease in CXCR3 surface expression. Thus, the expression levels of some activating and chemokine NK cell receptors are substantially modified during the infection of APC by a recombinant LASV harboring mutations affecting the exonuclease activity of NP.

NK cell activation mediated by rNP-LASV-infected APC involves cell contacts and soluble factors. We have previously shown that cell contacts as well as soluble factors are involved in NK cell activation mediated by LASV-infected Mϕ (15). Here, we tested whether cell contacts contribute to the activation and the modification of the repertoire of NK cells during rNP-LASV infection. NK cells were cultured for 72 h with rNP-LASV- or mock-

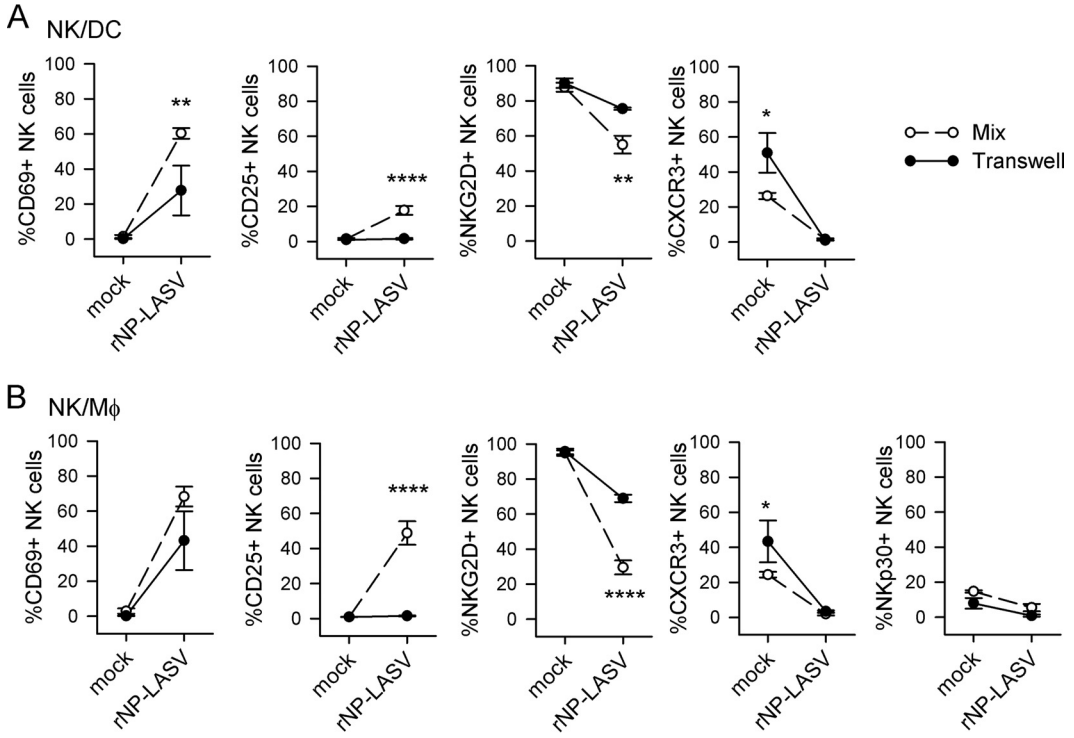

FIG 2 Cell contacts are involved in NK cell activation. Expression of surface molecules by NK cells cocultured with mock- or rNP-LASV-infected DC (A) or Mϕ (B). NK cells were cultured mixed with APC (Mix) or separated from APC by a semipermeable membrane (Transwell). NK cells were gated according to forward scatter/side scatter characteristics and expression of CD56. The percentage of NK cells expressing CD69, CD25, NKp30, NKG2D, and CXCR3 was analyzed at 72 h postinfection by flow cytometry; the values reported are means of three independent experiments. Standard errors of the means are represented by error bars. Two-way ANOVA followed by a Bonferroni *post hoc* test was used for statistical analysis, and significant differences between mix and transwell means are shown (*, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$).

infected APC either as mixed cultures or separated from them by a semipermeable membrane (transwell culture). NK cells were then stained and analyzed by flow cytometry. Consistent with previous findings, rNP-LASV-infected DC and Mϕ induced an increase in CD69 expression by NK cells in mixed cultures (Fig. 2). When NK cells were separated from the rNP-LASV-infected APC, CD69 was still upregulated but to a lower extent, which was significant for DC cultures, indicating that both cell contacts and soluble factors contribute to CD69 upregulation. In transwell cultures with no cell contacts between NK cells and rNP-LASV-infected APC, the CD25 upregulation observed in mixed cultures was completely absent. We also observed that the modification of the expression of NKG2D but not of CXCR3 was significantly smaller in transwell cultures than in mixed cultures. Thus, NK cell activation and the modification of the expression of some receptors involve cell contacts with infected DC and Mϕ as well as APC-produced soluble factors. However, the decline in CXCR3 expression is dependent only on soluble factors.

rNP-LASV-infected Mϕ induce the production of IFN-γ by NK cells. NK cells were cultured with rWT-LASV-, rNP-LASV-, or mock-infected DC or Mϕ, and IFN-γ synthesis was investigated. Total mRNA was extracted and analyzed by real-time RT-PCR; the changes in the abundance of IFN-γ mRNA in the NK/APC cultures during rWT-LASV infection were small, whereas there was a substantial and significant increase after stimulation with rNP-LASV-infected DC and Mϕ (Fig. 3A). As expected, we confirmed that low levels of mRNA encoding IFN-γ were tran-

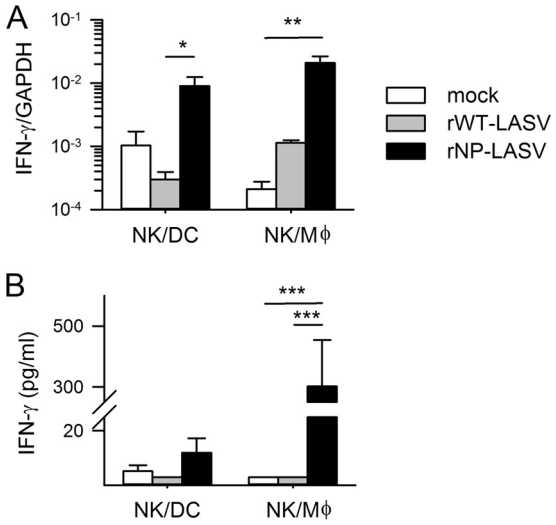

FIG 3 The infection of DC and Mϕ by rNP-LASV induces IFN-γ production by NK cells. Resting NK cells were cultured with mock-, rWT-LASV-, or rNP-LASV-infected DC or Mϕ. (A) IFN-γ mRNA was assayed at 24 h postinfection by quantitative RT-PCR, and values were normalized to the value for GAPDH. The results shown are means and standard errors of the means of independent experiments ($n = 4$ and $n = 5$ for NK/DC and NK/Mϕ experiments, respectively). (B) The supernatants from cultures of NK cells and mock-, rWT-LASV-, or rNP-LASV-infected DC or Mϕ were collected at 72 h postinfection and assayed for IFN-γ by ELISA. The results shown are means and standard errors of the means of independent experiments ($n = 5$ and $n = 7$ for NK/DC and NK/Mϕ experiments, respectively). The statistical significance of differences was determined using one-way ANOVA with a Bonferroni test (A) or Kruskal-Wallis with Dunn's test (B) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

scribed in DC and Mϕ single cultures (data not shown). Indeed, NK cells are known to be major producers of IFN-γ along with T cells. Culture supernatants were collected and assayed by ELISA. Large amounts of IFN-γ were detected only in NK cell and rNP-LASV-infected Mϕ cultures (Fig. 3B). This is the first demonstration that a recombinant LASV carrying mutations that affect the exonuclease function of NP can induce NK cell-mediated IFN-γ secretion in the presence of infected Mϕ.

Granzyme B, perforin, FasL, and TRAIL lytic factors as well as Fas receptor seem to be upregulated during rNP-LASV infection. DC and Mϕ were infected with rWT-LASV, rNP-LASV, or with mock supernatant, and NK cells were added to the cultures. We investigated the expression of some molecules involved in cytotoxicity, including granzyme B (GrzB) and perforin, components of lytic granules, and Fas/FasL and TRAIL/TRAIL receptors DR4 and DR5, involved in the death receptor (DR) pathway. The abundance of GrzB mRNA was higher, but not significantly higher, in culture with rNP-LASV-infected Mϕ than in controls (Fig. 4A). Similarly, the amount of intracellular GrzB found by flow cytometry was slightly, but not significantly, larger than that of controls in NK cells cultured with rWT-LASV-infected Mϕ only and rNP-LASV-infected APC (Fig. 4B). Only the amount of mRNA encoding perforin was found to be significantly above control values in the culture of NK cells and rNP-LASV-infected Mϕ (Fig. 4A). However, the amount of intracellular perforin in NK cells in the infected cultures was not different from that in controls (data not shown). FasL mRNA was more abundant in the culture of NK cells and rNP-LASV-infected Mϕ than in controls. The amount of TRAIL mRNA was unaffected by rWT-LASV infection but was substantially higher in cultures of NK cells and rNP-LASV-infected DC or Mϕ. Soluble FasL and TRAIL concentrations in the supernatant of rNP-LASV-infected NK/APC cultures were below the threshold of detection by ELISA (data not shown).

DC and Mϕ were harvested at 72 h postinfection and stained for the Fas receptor. No change was observed during rWT-LASV infection (Fig. 4C). However, the expression of Fas at the surface of rNP-LASV-infected DC and Mϕ was stronger although the difference was significant only for DC cultures. We found no difference in the expression levels of TRAIL receptors DR4 and DR5 (data not shown).

These investigations show that the infection by a recombinant LASV containing mutations affecting the exonuclease activity of NP seems to increase cytotoxic abilities of NK cells, possibly involving GrzB/perforin and Fas/TRAIL death receptor pathways.

rNP-LASV-infected APC stimulate NK cell cytotoxicity. NK cells were cultured with rWT-LASV-, rNP-LASV-, or mock-infected DC or Mϕ for 48 h. K562 cells lacking MHC class I were added to the culture for the last 5 h to trigger NK cell-mediated killing. NK cells were then stained, and degranulation was analyzed by CD107a surface exposure. The numbers of CD107a-expressing NK cells increased after dual stimulation with rWT-LASV-infected Mϕ and K562 target cells, whereas there was no change with infected DC (Fig. 4D). NK cell degranulation toward K562 cells was increased after stimulation with rNP-LASV-infected DC and Mϕ, but the difference was significant only for DC cultures. Even in the absence of sensitive K562 cells, the expression of CD107a at the surface of NK cells increased upon rNP-LASV infection of DC and Mϕ; this indicates that NK cells can themselves trigger degranulation toward infected APC (Fig. 4E). APC were infected with rNP-LASV at a low MOI to analyze viral growth, and NK cells were added to the cultures. As previously shown (19), rNP-LASV is strongly attenuated, and the titers were not detectable after 24 to 48 h postinfection (data not shown). Unfortunately, we could not evaluate any difference in rNP-LASV replication in the presence or absence of NK cells. These experiments show that DC and Mϕ infected by a recombinant LASV mutated within or nearby the exonuclease domain of NP can increase NK cell cytotoxic functions and stimulate NK cell killing.

rNP-infected DC and Mϕ express NK cell-activating NKG2D ligands. DC and Mϕ were infected with rWT-LASV, rNP-LASV, or a mock supernatant and cultured with or without NK cells. The expression levels of NKG2D ligands, including MHC class I-related chain A (MICA) and MICB, UL16-binding protein 1 (ULBP-1) and ULBP-2, and B7H6, an NKp30 ligand, were investigated by quantitative RT-PCR and flow cytometry. During rWT-LASV infection, only Mϕ seemed to induce MICB transcription (Fig. 5A). The amounts of the mRNAs for MICA and MICB were modestly higher in the cultures of NK cells and r-NP-LASV-infected DC and Mϕ than in controls; the differences were significant only for MICB mRNA in NK/Mϕ cultures. We observed similar results in the absence of NK cells (data not shown). MICA/B molecules were significantly overexpressed at the surface of r-NP-LASV-infected DC and Mϕ, whereas there was no difference during rWT-LASV infection (Fig. 5B). The amounts of ULBP-1 and ULBP-2 mRNAs were not modified in the culture of NK cells and rWT-LASV-infected APC (Fig. 5A). However, there were significantly more ULBP-1 and ULBP-2 mRNAs in NK/DC and NK/Mϕ cultures upon rNP-LASV infection than in controls. The amount of soluble ULBP-1 in the supernatant of NK/APC during rWT-LASV or rNP-LASV infection was not different from that in mock cultures, as determined by ELISA (Fig. 5C). There was significantly less soluble ULBP-1 in the supernatant of NK/Mϕ cultures when Mϕ were infected with rNP-LASV than with rWT-LASV. B7H6 mRNA was slightly but not significantly higher during rNP-LASV infection than under other conditions only in NK/Mϕ cultures (Fig. 5A). The mRNA for BAT3/BAG6, another NKp30 ligand, was not modified after infection (data not shown). Thus, infection with a recombinant LASV that contains mutations affecting the exonuclease function of NP upregulates some NKG2D ligands at the surface of APC.

rNP-LASV-infected APC produce mRNAs encoding cytokines involved in NK cell activation. DC and Mϕ were infected with rWT-LASV, rNP-LASV, or a mock supernatant and cultured with or without NK cells. Total RNAs were extracted 24 h later, and mRNAs encoding cytokines involved in NK cell activation were assayed by quantitative RT-PCR. We found no significant modification of the levels of mRNAs encoding IL-12p35, IL-12p40, IL-15, and IL-18 during rWT-LASV infection of DC or Mϕ (Fig. 6A). In contrast, these mRNAs were more abundant in the rNP-infected DC and Mϕ cultures. Similarly, IL-12p35 and IL-15 mRNAs were significantly more abundant in all NK/APC cultures upon rNP-LASV infection than in controls. The mRNAs for IL-12p40 and IL-18 were also more abundant when NK cells were added to rNP-LASV-infected Mϕ but were unaffected in the NK/DC cultures. Neither IL-12 nor IL-15 was detected in the supernatant of infected cultures (data not shown), and the amounts of IL-18 were similar in all samples (Fig. 6B), indicating that these cytokines were not released in large amounts. These analyses show that infection by a recombinant LASV mutated within or nearby

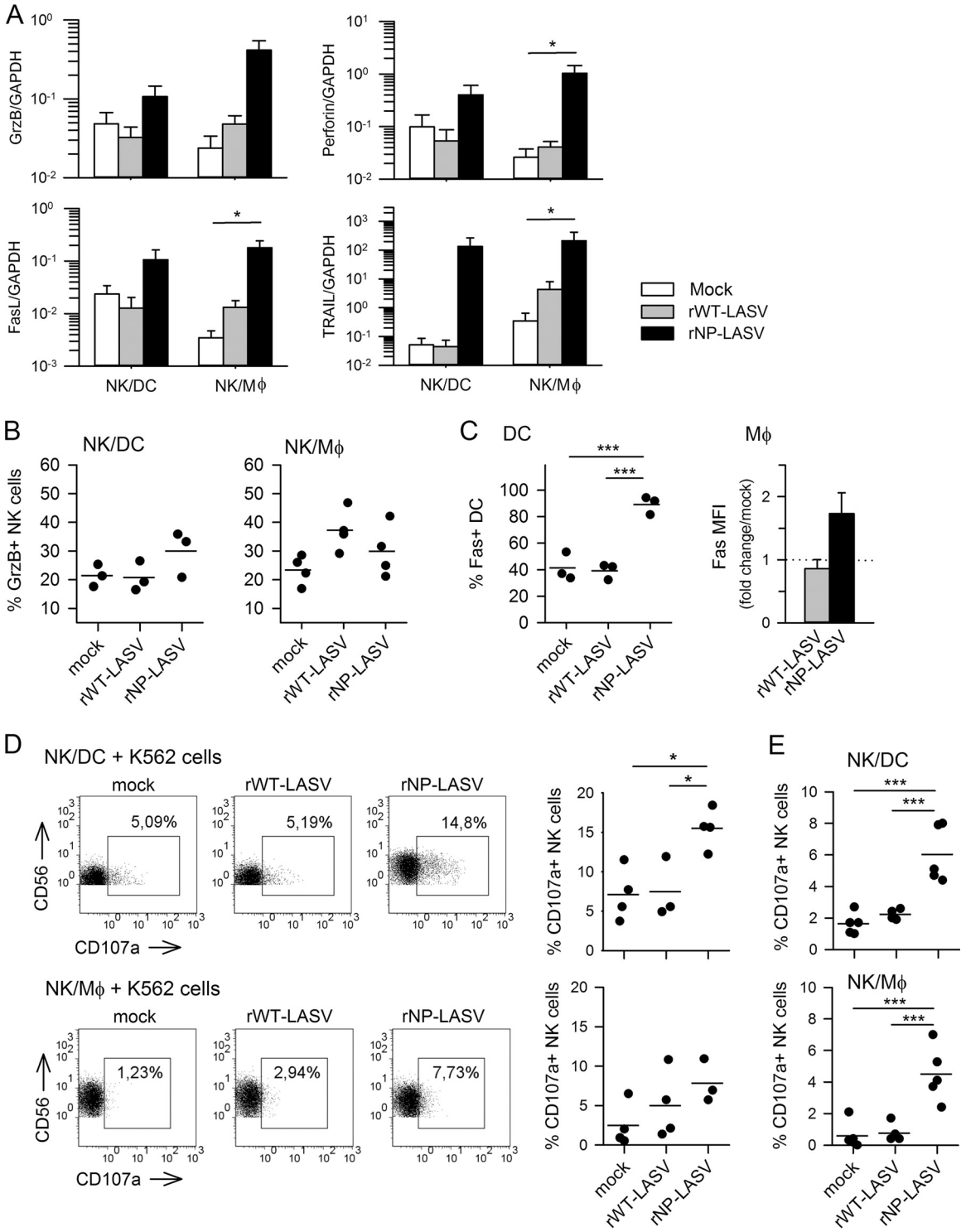

FIG 4 The infection of DC and Mϕ by rNP-LASV induces increased cytotoxicity of NK cells and the killing of infected cells. Resting NK cells were cultured with mock-, rWT-LASV-, or rNP-LASV-infected DC or Mϕ. (A) The mRNAs encoding GrzB, perforin, FasL, and TRAIL were assayed in cocultures of NK cells and mock-, rWT-LASV-, or rNP-LASV-infected DC or Mϕ at 24 h postinfection by quantitative RT-PCR. The values were normalized to those for GAPDH, and the results shown are means and standard errors of the means of independent experiments ($n = 4$ and $n = 5$ for NK/DC and NK/Mϕ experiments, respectively). (B) Intracellular GrzB in NK cells cocultured with DC or Mϕ and analyzed at 72 h postinfection by flow cytometry. NK cells were gated according to forward scatter/side scatter characteristics and expression of CD56. Dots indicate the percentages of positive cells in independent experiments, and mean values are indicated by horizontal bars. (C) Expression of Fas by DC and Mϕ determined at 48 h postinfection by flow cytometry. The percentages of positive DC are depicted as dots corresponding to independent experiments, and their means are indicated by horizontal bars (left panel). The mean fluorescence intensity (MFI) of Fas was analyzed for Mϕ, and results are shown for rWT-LASV- and rNP-LASV-infected Mϕ as fold change relative to mock-infected cells (right panel). Means and standard errors of the means of three independent experiments are shown. (D) K562 target cells were added to NK/DC and NK/Mϕ cultures at 48 h

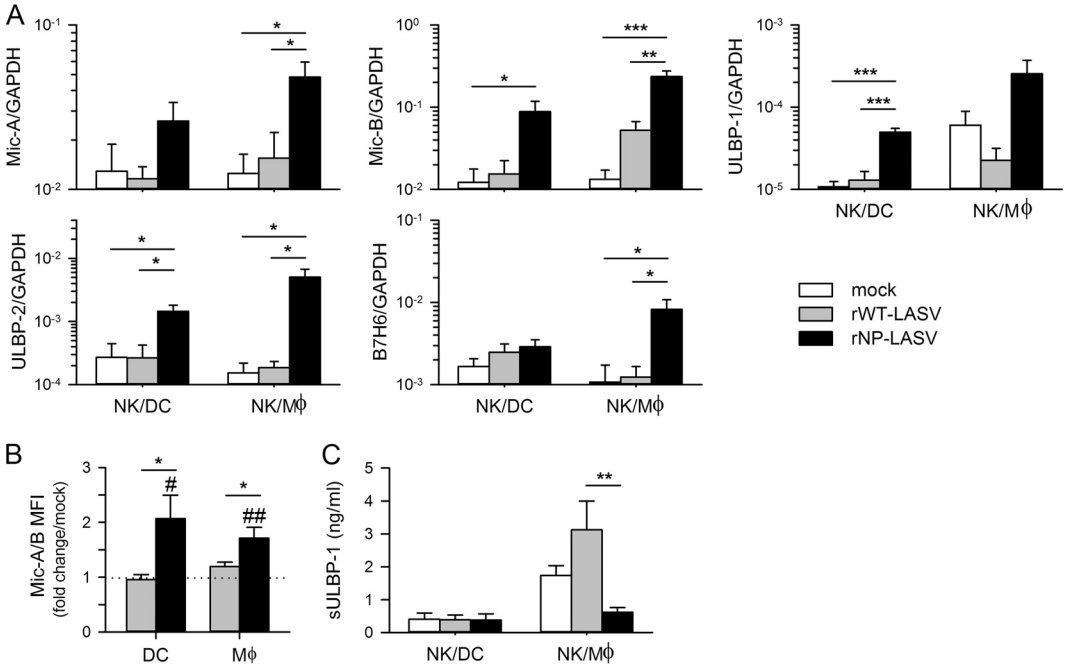

FIG 5 The infection of DC and Mϕ by rNP-LASV induces modification of the expression of NKG2D and NKp30 ligands. DC and Mϕ were infected by rWT-LASV or rNP-LASV or mock supernatant and cultured with or without NK cells. (A) The mRNAs encoding MICA, MICB, ULBP-1, ULBP-2, and B7H6 were assayed at 24 h postinfection by quantitative RT-PCR. The results were normalized to the values for GAPDH and are shown as means and standard errors of the means of independent experiments ($n = 4$ and $n = 5$ for NK/DC and NK/Mϕ experiments, respectively). (B) DC and Mϕ were stained with specific MICA/B antibody at 24 and 48 h postinfection, and mean fluorescence intensity (MFI) was determined by flow cytometry. Results are shown as fold change relative to mock-infected cells. Means and standard errors of the means of seven independent experiments are shown, and significant differences between rWT-LASV and rNP-LASV conditions (*) or mock and rNP-LASV (#) are indicated. (C) The supernatants of NK/DC and NK/Mϕ cultures were collected at 72 h postinfection, and the presence of soluble ULBP-1 was analyzed by ELISA. The results reported are means and standard errors of the means of independent experiments ($n = 5$ and $n = 7$ for NK/DC and NK/Mϕ experiments, respectively). Statistical significance of differences was determined using one-way ANOVA followed by a Bonferroni *post hoc* test (* and #, $P < 0.05$; ** and ##, $P < 0.01$; ***, $P < 0.001$).

the exonuclease domain of NP leads to the transcription of the genes for IL-12p35, IL-12p40, IL-15, and IL-18 in DC and Mϕ.

DISCUSSION

The pathogenesis and the immune responses occurring during viral hemorrhagic fevers including LASV infection are still poorly understood. Nevertheless, the etiologic agents currently cause major public health problems in countries of endemicity, and some viruses are even emerging in new, populated areas (20). The primary type I IFN response is inhibited in APC by LASV NP (7, 8), suggesting that the overall immune response may be affected during the infection. Our work with an *in vitro* model previously showed that NK cells are activated by LASV-infected Mϕ but not by LASV-infected DC. However, these cells are not able to trigger effective cytotoxicity toward infected cells nor secrete IFN-γ (15). Here, we show that a recombinant LASV carrying two mutations affecting the exonuclease activity and IFN suppression of NP can mediate DC and Mϕ activation and functional NK responses. Importantly, DC were not inhibited after infection with this mutant, and the activation of Mϕ triggered by this mutant is greater than that with rWT-LASV infection. NK cells secreted IFN-γ after stimulation by rNP-LASV-infected Mϕ, and NK cell cytotoxicity toward sensitive K562 cells and infected APC was increased. Cell contacts with APC are essential for NK cell activation and modulation of the repertoire of receptors, but soluble molecules, including cytokines secreted by APC, also seem to play a role. These observations confirm that the exonuclease function of LASV NP is essential for immune suppression and the inhibition of functional NK cell responses.

We show that NK cell activation is mediated by cellular contact with infected APC and soluble factors produced by these accessory cells. APC-mediated NK cell activation is well documented, and it involves activating receptor/ligand signaling pathways (18). We show that rNP-LASV induces a change in the phenotype of infected APC: first, some NK cell-activating ligands are expressed at the surface of APC following rNP-LASV infection; then, the expression of several genes encoding cytokines important for NK cell activation is also modulated. In addition, we have also ob-

postinfection, and the expression of surface CD107a was determined by flow cytometry. NK cells were gated according to forward scatter/side scatter characteristics and expression of CD56. Representative dot plots for four independent experiments are shown. In addition, the percentages of positive cells from independent experiments ($n = 3$ or 4) are displayed as dots, and mean values are indicated. (E) The expression of surface CD107a by NK cells cultured with DC or Mϕwas analyzed at 48 h postinfection by flow cytometry. The results are depicted as percentages of positive cells from independent experiments ($n = 5$) displayed as dots, and means are indicated by horizontal bars. Statistical significance of differences was determined using Kruskal-Wallis followed by Dunn's test (A) or one-way ANOVA with a Bonferroni test (B, C, D, and E) (*, $P < 0.05$; ***, $P < 0.001$).

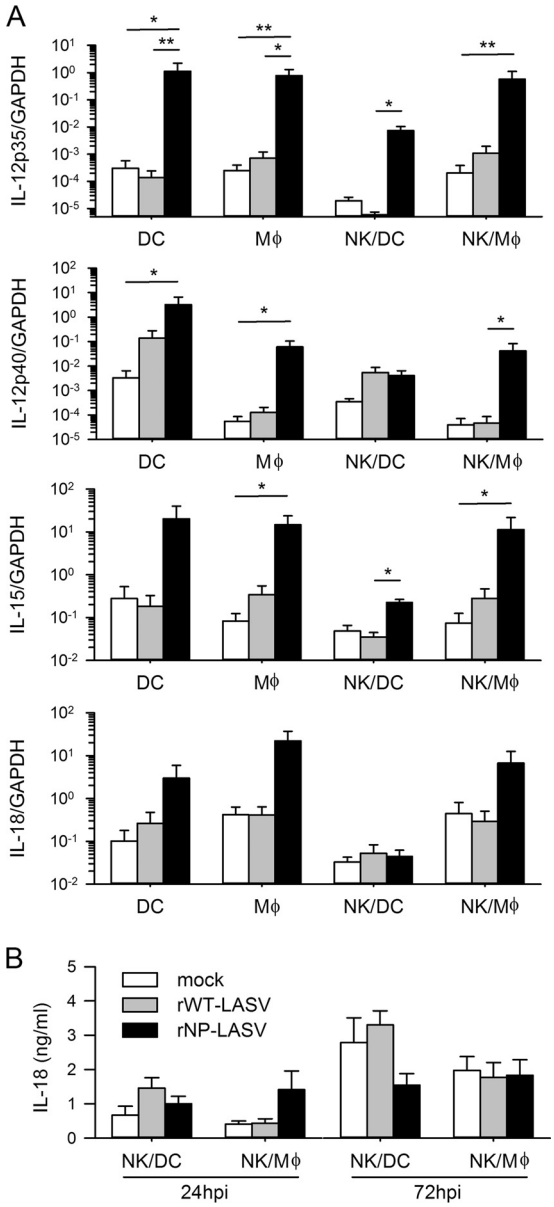

FIG 6 The infection of DC and Mϕ by rNP-LASV induces the production of cytokine mRNA. DC and Mϕ were infected with rWT-LASV or rNP-LASV or mock supernatant and cultured with or without NK cells. (A) The mRNAs encoding IL-12p35, IL-12p40, IL-15, and IL-18 were assayed at 24 h postinfection by quantitative RT-PCR. The results were normalized to the values for GAPDH and are shown as means and standard errors of the means of four to seven independent experiments. (B) The supernatants of NK/DC and NK/Mϕ cultures were collected at 24 and 72 h postinfection and assayed for IL-18 by ELISA. The means and standard errors of the means of four to seven independent experiments are reported. Statistical significance of differences was determined using Kruskal-Wallis test followed by Dunn's *post hoc* test (*, $P < 0.05$; **, $P < 0.01$). hpi, hours postinfection.

served that rNP-LASV-infected DC and Mϕ are activated in that costimulatory molecules are upregulated and cytokines and chemokines are released (35). The NK cell activation that we report here is thus consistent with an activated phenotype of APC induced by rNP-LASV. The role of the interactions between APC and NK cells in the overall immune response including T cell immunity during rNP-LASV infection, however, has still to be determined.

Further illustration of the change in NK cell phenotype during rNP-LASV infection is the downregulation of CXCR3. This is likely due to desensitization of the receptor following the binding of CXCL9, CXCL10, and CXCL11; indeed, these chemokines are produced in large amounts during rNP-LASV infection (21). This mechanism could trigger NK cell migration. NK cell depletion from the blood has been observed during LASV infection of nonhuman primates (22) and may be due to NK cell migration following CXCR3 downregulation. *In vivo* studies are needed to document this issue.

NKp30- and NKG2D-expressing NK cell populations decrease after rNP-LASV infection, and this phenomenon has been observed during NK cell activation (23, 24) and following activation by exogenous IL-2/phytohemagglutinin (PHA) (15). NKp30 and NKG2D are cytotoxicity receptors involved in the recognition of target cells (25). The downregulation of these two receptors at the surface of NK cells may be due to the binding of activating ligands followed by desensitization. Specifically, in response to activating stimuli, macrophages express various ligands of the activating receptor NKG2D, including ULBP-1, ULBP-2, ULBP-3, and MICA (24). Consistent with this, we show that MICA/B are expressed at the surface of DC and Mϕ after rNP-LASV infection. Other NKG2D ligands such as ULBP molecules may also play a role. In accordance with these findings, prolonged exposure to its ligands downregulates NKG2D on NK cells (23). We did not find any change in the expression of the published NKp30 ligands, BAT3 and B7-H6. Unfortunately, little is known about the identity of NKp30 ligands, and some as yet unidentified molecules may be involved during rNP-LASV infection. We believe that soluble ligands of activating NK cell receptors may play a role in desensitizing these receptors and more generally in the modulation of NK cell phenotype. Intracellular BAT3 can be released in exosomes by DC and bind to NKp30, thereby triggering NK cell functions (26). Similarly, NKG2D ligands including ULBP molecules are shed in response to HIV-1 and inhibit NKG2D-mediated NK cell activation (27). Further investigation of the expression of such soluble ligands of NK cell-activating receptors during LASV infection would be informative. Finally, the results of our study of rNP-LASV infection are consistent with published findings that activating ligands are produced by activated APC and mediate NK cell activation.

One of the most striking differences between NK cell activation mediated by rNP-LASV and that mediated by LASV is the ability of NK cells to lyse rNP-LASV-infected APC. Indeed, despite substantial acquisition of cytotoxic functions and lysis of K562 cells, NK cells failed to lyse LASV-infected Mϕ, which thus appear to be resistant to death mediated by activated NK cells. In contrast, NK cells efficiently degranulated toward rNP-LASV-infected APC, indicating that they are able to kill both DC and Mϕ during infection by rNP-LASV. This suggests that NK cells have acquired a more efficient cytotoxic phenotype and/or that rNP-LASV-infected APC are susceptible to NK cell-mediated cell death. Further work is required to characterize the mechanisms underlying these differences. Nevertheless, the exonuclease activity of LASV NP to affect substantially the functional properties of NK cells and/or to render infected cells resistant to NK cell-mediated cell death is probably a key event in immunosuppression during Lassa fever. Unfortunately, we were not able to detect any difference between

the titers of the supernatants of NK/APC cocultures and APC single cultures as NP-LASV replicates poorly in DC and Mϕ. However, we demonstrate that activated NK cells are fully functional and kill rNP-LASV-infected APC in the culture. Whether they may be involved *in vivo* to control the infection at an early step during rNP-LASV infection or after challenge with LASV has to be evaluated in further studies.

Functional NK cells also secrete IFN-γ. We show here that rNP-LASV induces substantial production of IFN-γ in NK/Mϕ culture. In our model, IFN-γ secretion can be modulated by the levels of type I IFN as described during lymphocytic choriomeningitis virus infection (28, 29). Specifically, IFN-γ production is regulated by a balance between STAT1 and STAT4 transcriptional pathways that are differentially activated according to the levels of IFN-α/β. NK cells express high levels of STAT4, driving IFN-γ secretion in response to type I IFN, and STAT1 activation represses IFN-γ production. Nothing is known about STAT1/STAT4 regulation in NK cells during LASV infection. Further studies are needed to determine if, in our model, the expression of IFN-γ by NK cells is modulated by the type I IFN response through these transcription factors. Other cytokines may play a role in IFN-γ production: IL-12 and IL-18 are important mediators of IFN-γ production (18, 30, 31) and are likely to be involved during rNP-LASV infection. We show that IL-12 and IL-18 mRNAs are produced in NK/APC cocultures during rNP-LASV infection and also that these cytokines themselves were not detectable in the supernatants. Possible explanations include a local secretion or a contact-dependent mechanism enabling NK cells to directly use these cytokines. Indeed, IL-12 can transit from DC to NK cells via synapses (30). Such a mechanism may operate in our model although this remains to be demonstrated. Also, the role of IFN-γ in the initiation of T cell responses during rNP-LASV infection remains unclear; we do not know whether IFN-γ is produced *in vivo* during rNP-LASV infection. Further work on these issues may indicate whether rNP-LASV-activated NK cells could mediate protection against WT-LASV.

LASV and other arenaviruses suppress IFN production via a unique exonuclease activity of NP (7–9). This function allows NP to digest viral dsRNA substrates generally sensed by retinoic acid-inducible gene I (RIG-I). The mechanism leading to the inhibition of the primary type I IFN response during LASV infection is still unclear. As shown for lymphocytic choriomeningitis virus (32), another Old World *Arenavirus*, this mechanism may involve the RIG-I/melanoma-differentiation-associated gene 5 (MDA5) pathway. We show that residues 389 and 392 in the active site of the exonuclease domain of NP or nearby strongly affect IFN production by APC (19). Other investigators have confirmed that residue 389 is mandatory for functional exonuclease activity of NP (7, 9). In addition to the inhibition of primary responses in LASV-infected cells, an early blockade of type I IFN production prevents subsequent transcription of IFN-stimulated genes (ISG), including some encoding mediators of NK cell activation. Our results here suggest that the lack of functional activity of NK cells observed during WT-LASV infection (15) is due at least partially to the inhibition of the type I IFN response via the enzymatic activity of LASV NP. The amounts of IFN-α and IFN-β may not be sufficient to induce fully activated APC and NK cells. In contrast, rNP-LASV-infected DC and Mϕ produce large amounts of type I IFN, thus driving NK cell activation. The role of these mediators in rNP-LASV infection should be investigated. Unfortunately, neutralizing antibodies directed against IFN-α and its receptor were insufficient to block the amounts of type I IFN present in our model (data not shown). Also various other investigative approaches are problematic with primary cells used as in our model. It is unlikely that the induction of NK cell responses directly results from a low replication of rNP-LASV in DC and Mϕ. In contrast to other studies (4, 7), we previously reported that rNP-LASV is stable but has a reduced minigenome activity, suggesting that residues D389 and G392 play a crucial role in viral infectivity and replication (19). It has been shown that the lymphocytic choriomeningitis virus, an Old World *Arenavirus*, harboring a D382 substitution in NP (equivalent to 389 in LASV NP) grows to a slightly decreased level in IFN-deficient cells and is dramatically attenuated in IFN-competent cells (4). Moreover, IFN-α has been shown to regulate LASV replication (33). Together, these data suggest that the exonuclease activity upstream to the anti-IFN function of LASV NP is required for replication in APC, but we believe that it is mostly due to an indirect effect including a strong type I IFN response. NK cell responses will then result from rNP-LASV-induced APC activation.

A recent study showed that LASV and other arenavirus NPs inhibit the translocation and transcriptional activity of nuclear factor-κB (NF-κB) (34). Moreover, the authors reported that recombinant lymphocytic choriomeningitis virus containing D382A and G385A substitutions (corresponding to 389 and 392 in LASV sequence, respectively) failed to inhibit NF-κB activation. Such findings have to be demonstrated for LASV, but D389A and G392A substitutions in LASV NP are likely to be crucial in the inhibition of NF-κB transcriptional activation. We believe that rNP-LASV may thus allow NF-κB-triggered immune responses in our model. This explains the substantial amounts of inflammatory chemokines secreted by rNP-LASV-infected APC (21).

In conclusion, we show here that residues 389 and 392 are involved in APC-mediated NK cell activation. Mutations at these positions affect NP function leading to the induction of immune responses. These findings thus confirm that the exonuclease activity of LASV NP is crucial in the inhibition of the immune response during LASV infection: it acts on APC with consequences for NK cell responses. As the infection with rNP-LASV leads to APC activation and NK cell responses presenting an activated phenotype and a full spectrum of functional properties, we would expect than T cell responses would similarly be boosted. This work provides new tools and suggests significant possibilities relevant to the immune response during LASV infection and viral hemorrhagic fevers generally. It also reveals potentially important clues about the mechanisms by which these viruses evade the immune system.

ACKNOWLEDGMENTS

M.R. held a fellowship from the Délégation Générale de l'Armement (G. Vergnaud, the French Army).

We thank S. Becker for providing us with the AV strain of LASV, M. Bouloy for LASV reverse genetic tools, T. G. Ksiazek, P. E. Rollin, and P. Jahrling for LASV MAbs, and the Etablissement Français du Sang for providing human blood. We also thank the Laboratoire P4-Jean Mérieux team for access to their BSL4 facility.

We declare that we have no conflicts of interest.

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